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Original Article

Comparison of IS6110 and 'short fragment' *devR* (Rv3133c) gene targets with phenotypic methods for diagnosis of *Mycobacterium tuberculosis*

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ABSTRACT

Background: Tuberculosis (TB), declared a global emergency in 1993 by the WHO, remains a worldwide public health problem. Rapid diagnosis is required for treatment and prevention. This study compares genotypic methods using two gene targets [IS6110 and 'short fragment' *devR* (Rv3133c)] with phenotypic methods [Lowenstein Jensen (LJ) and BACTEC 460] for the diagnosis of TB while using Ziehl Neelsen (ZN) staining as the gold standard. **Methods:** 56 clinical TB samples from a tertiary care apex center along with 50 healthy control samples, excluding samples from patients already on ATT were processed by routine USP methodology. Smears were graded by ZN stain. Solid media (LJ) and liquid media (BACTEC 460) were used along with IS6110 and 'short fragment' *devR* (Rv3133c) specific gene amplification and comparatively analyzed.

Results: 50/56 samples were positive by phenotypic methods, 53 by IS6110 and 45 by *devR* (Rv3133c) amplification. 38 samples were positive by both phenotypic and genotypic methods. IS6110 detected six and *devR* (Rv3133c) detected five phenotypically negative samples. Both IS6110 and *devR* (Rv3133c) were positive in 42 samples. 11 *devR* (Rv3133c) negative samples were positive by IS6110 and three IS6110 negative samples were positive by 'short fragment' *devR* (Rv3133c). Compared to phenotypic methods, the sensitivity, specificity, positive and negative predictive values of IS6110 was 94%, 89.29%, 88.68% and 94.34% while that of *devR* (Rv3133c) was 80%, 91.07%, 88.89% and 83.61% respectively.

Conclusion: Simultaneous use of both phenotypic and genotypic methods increases the yield of positive results.

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Introduction

Tuberculosis (TB) remains a worldwide public health problem even though *Mycobacterium tuberculosis* (MTB) was discovered

120 years ago. Despite active anti-tubercular therapy (ATT) and National TB control programs, the containment of the disease is a big challenge. Both the pathogen and disease are sinister. MTB is endemic and is usually transmitted by aerosol,

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droplet infection with ID₅₀ < 10 bacilli. It is a facultative intracellular organism resisting intracellular killing and has evolved into multi drug resistant (MDR), extremely drug resistant (XDR) and even totally drug resistant (TDR) strains.¹ The disease has varied incubation period, clinical features, indolent course and requires prolonged treatment. The problem is further magnified by the fact that patients often initially report late to the hospital and drug compliance is poor.

In 1993, WHO unprecedentedly declared TB as a global emergency. In 2006, WHO estimated 14.4 million (219/100,000 population) total cases, 9.2 million (139/100,000 population) new TB cases, 4.1 million (62/100,000 population) new smear positive cases, 789,000 (7.7%) HIV-TB co-infected cases with 1.7 million (925/100,000 population) deaths including 231,000 deaths in HIV-TB coinfections.^{2,3} Developing world, especially South East Asia, Western Pacific and Africa bear 95% of the TB burden. The contributing factors are higher endemicity, overcrowding, poor socioeconomic conditions, decaying public health infrastructures, increased drug resistance and growing HIV-AIDS population. India shares one third of this burden, with 30% prevalence, 2.2 million annual cases, 1 million new smear positive cases and an infection rate of 1–2%.^{4,5}

Rapid diagnosis is the keystone for early treatment and reduction of disease burden. Smear microscopy and culture methods [Lowenstein Jensen (LJ), Middlebrook] are complemented by both phenotypic and genotypic methods. The phenotypic methods include Mycobacterial growth indicator tube, Septi-check, MB-Bact System, beta-galactosidase expression, E-test, PhaB, Alamar Blue, Luciferase Reporter Phage, flowcytometry assays etc. The genotypic methods include polymerase chain reaction (PCR), PCR-SSCP, PCR-Heteroduplex formation, Ligase chain reaction, Line probe assay, DNA Microarrays, dideoxy fingerprinting, hybridization protection assays, automated DNA sequencing and Loop mediated isothermal amplification. The diagnosis by routine culture based methods takes about 3–8 weeks on agar based Middlebrook 7H10/7H11 or egg based LJ and about 3 weeks in liquid 7H9 medium. BACTEC is a liquid based culture method detecting growth index in about 14 days. The genotypic tests take few hours, are highly sensitive and specific and especially useful in cases of tubercular meningitis.^{6,7} Major disadvantages include requirement of robust infrastructure, inherent PCR associated problems and need to complement them with culture based tests.

This study compares and evaluates genotypic methods using two gene targets [IS6110 and 'short fragment' *devR* (Rv3133c)] with phenotypic methods based on solid and liquid culture media (LJ and BACTEC 460) for the diagnosis of TB while using Ziehl Neelsen (ZN) staining method as the gold standard.

Materials and methods

56 clinical TB samples from a tertiary care apex center along with 50 control samples, excluding samples from patients already on ATT were processed by the USP methodology⁸ after approval from the Hospital Ethical Committee. The sample was mixed with a solution containing 5 M

guanidinium hydrochloride, 2 M Tris-HCl (pH 7.5), 0.5 M EDTA (pH 8.0), 20% Sarkosyl, β-mercaptoethanol (all from Sigma-Aldrich Corporation, St. Louis, Missouri 63103, USA), incubated for 10–15 min at room temperature, centrifuged, supernatant decanted, pellet resuspended in Tween 80 solution and transferred to a 1.5 ml polypropylene lead vial. Smears were prepared for ZN (carbol fuchsin, 3% HCl in 95% ethanol, methylene blue) and the smears were graded 1+ (1–9 bacilli/100 fields) to 4+ (>9 bacilli/field). LJ slants and Middlebrook 7H9 media in BACTEC 460 (Becton, Dickinson and Company, Sparks, MD 21152, USA) were inoculated and incubated at 37 °C for up to 8 weeks. For BACTEC, 500 µg N acetyl-L-cysteine and phosphate buffer were added to a 100 ml solution containing 50 ml each of 4% NaOH and 2.9% sodium citrate, vortexed, centrifuged, supernatant decanted, pellet resuspended in buffer, PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin) added and incubated for 3 days at 37 °C. Growth index (more than 10 as significant) was measured on 4th, 7th and 11th/12th day.

For genotypic assays, the resuspended material was centrifuged, supernatant decanted, mixed with lysis solution containing Chelex 100, distilled water, Triton X and Tween 80, heated at 90–95 °C, centrifuged and supernatant was used. IS6110 specific PCR was performed in 40 µl reaction mixture containing a 0.5 µM concentration (each) of forward primer IS6110 (5'-CCTGCGAGCGTAGGCGTCGG-3') and reverse primer IS6110 (5'-CTCGTCCAGCGCCGCTTCGG-3'), 1x PCR buffer with (NH₄)₂SO₄ (MBI Fermentas, Vilnius, Lithuania), 1.5 mM MgCl₂, 0.2 mM concentration of each deoxynucleoside triphosphate (dNTP), 1 U Taq DNA polymerase, water and 10 µl specimen DNA.⁹ Denaturation was carried at 94 °C for 10 min, annealing at 94 °C for 1 min (45 cycles), extension at 60 °C for 1 min 30 s and final extension at 72 °C for 7 min. Short fragment *devR* (Rv3133c) specific PCR was performed in 40 µl reaction mixture containing 0.5 µM concentration (each) of forward primer *devR* (Rv3133c) f2 (5'-TGGCAACGGCA-TTGAAGTGT-3') and reverse primer *devR* (Rv3133c) r2 (5'-TAAGCAGGCCAGTAGCGT-3'), 1x Taq Polymerase Buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1 µl/40 µl Taq DNA Polymerase, water and 10 µl specimen DNA.¹⁰ Denaturation was carried at 94 °C for 10 min, annealing at 52 °C for 1 min (45 cycles), extension at 72 °C for 30 s and final extension at 72 °C for 7 min. 10 µl water as negative control and 5 µl MTB DNA (2–5 ng) as positive control were put for both PCRs. PCR products [IS6110-123 bp, *devR* (Rv3133c)-308 bp] were visualized by ethidium bromide staining on a 2.3% agarose gel by electrophoresis (Figs. 1 and 2). Sample DNA was spiked with 1–2 ng pure MTB DNA to check PCR inhibition. Internal quality control measures and good laboratory practices were followed at all steps. Descriptive statistics and Kappa analysis were used to analyze data.

Results

The age and sex distribution is depicted in Table 1. 50/56 samples were positive by phenotypic methods (ZN staining, culture on LJ and BACTEC 460), 53 by IS6110 and 45 by 'short fragment' *devR* (Rv3133c) gene amplification. Table 2 shows

Fig. 1 – *IS6110* assay for diagnosis of MTB.

the distribution of positive samples. 38 samples were simultaneously positive by both phenotypic and genotypic methods [*IS6110* and 'short fragment' *devR* (*Rv3133c*)]. *IS6110* detected 6 and 'short fragment' *devR* (*Rv3133c*) detected 5 smear/LJ/BACTEC negative samples. Concordance of genotypic results was seen in 42 samples where both *IS6110* and 'short fragment' *devR* (*Rv3133c*) were positive. 11 'short fragment' *devR* (*Rv3133c*) negative samples were positive by *IS6110* and 3 *IS6110* negative samples were positive by 'short fragment' *devR* (*Rv3133c*). Compared to phenotypic methods, the sensitivity, specificity, positive and negative predictive values (PPV and NPV) of *IS6110* was 94%, 89.29%, 88.68% and 94.34% respectively while that of 'short fragment' *devR* (*Rv3133c*) was 80%, 91.07%, 88.89% and 83.61% respectively. The strength of agreement was perfect with *IS6110* and moderate with 'short fragment' *devR* (*Rv3133c*) when compared to phenotypic methods by kappa statistical analysis.

Discussion

ZN stain is a simple, rapid, inexpensive method with specificity of 98%. The disadvantage is it cannot discriminate between MTB and non tuberculous mycobacteria (NTM) species and lacks sensitivity (5000-10,000 bacilli/ml are required for positive result). Solid media like LJ and Middlebrook can detect ≤ 10 bacilli and identify species. They are also useful in testing drug susceptibility and response to therapy. A major disadvantage is slow growth rate of MTB which delays diagnosis.

Fig. 2 – *devR* (*Rv3133c*) assay for diagnosis of MTB.

Table 1 – Age and sex of patients.

Age/sex	No. of cases	% Age
1–10 yrs	2	3.571%
10–20 yrs	3	5.3%
20–30 yrs	15	26.78%
≥40 yrs	36	64.2%
Males	41	73.21%
Females	15	26.7%

BACTEC 460 uses Middlebrook 7H9 broth base, bovine serum albumin, casein hydrolyzate, catalase and polyoxyethylene stearate as growth enhancers, small quantities of PANTA to suppress contaminants and ^{14}C labeled palmitic acid as the growth detector. Traces of $^{14}\text{CO}_2$ released are quantitatively detected and translated to a numerical value (Growth Index) by a sensitive radioactive detector system based on electrical impedance. In 12–13 days, 200 viable MTB can be detected whereas in 14–17 days, even 20 viable bacilli can be detected.^{11,12} BACTEC 460 reduces identification time, gives increased yield, performs susceptibility studies and differentiates NTM by adding p-nitro-alpha acetyl amino-beta hydroxyl propiophenone (NAP) to blood culture vials as MTB and *Mycobacterium bovis* cannot grow in NAP containing media. However, it uses expensive instruments, is unable to process specimens without NALC–NaOH, may give mixed growth including contaminants, doesn't characterize colony morphology and requires handling of radioactive materials and needles.¹¹ ZN stain on BACTEC media is required to exclude other bacteria breaking through antibiotic inhibition. Pseudomycobacterial infections due to incomplete sterilization of needles have been reported.^{6,7,12,13}

Genotypic assays are purported to evolve as the new gold standard in MTB diagnosis. They can detect MTB in smear/culture negative specimens and non-cultivable bacteria from patients under therapy till 5–11 months.¹⁴ Numerous gene targets like *IS6110*, *devR* (*Rv3133c*), 65 kDa, *TRC4*, *GCRS*, *MTB40*, *MPB-64*, *Del portillo* (396bp), *Altarmirano* (969 bp) etc have been developed, but their widespread usage awaits detailed evaluation.¹⁵ *IS6110*, amplifying 123 bp fragment, is a transposon which is self replicating DNA specific to MTB complex. While *IS6110* often repeats 20 times in MTB, it may be missing or present as a single copy in a significant proportion of clinical

Table 2 – Distribution of samples.

Sample	ZN/LJ/BACTEC	<i>IS6110</i>	<i>devR</i>
Pus	13	13	12
Synovial fluid	11	12	11
Pleural fluid	9	9	5
Ascitic fluid	4	4	5
Bronchoalveolar lavage	5	4	4
CSF	1	3	3
Bone marrow	2	2	1
Tissue	4	4	3
Peritoneal fluid	Nil	1	1
Pericardial fluid	1	1	Nil
Total	50	53	45

isolates from India.¹¹ Short fragment *devR* (Rv3133c) is more sensitive and detects 250–500 genome equivalents while 'long fragment' *devR* (Rv3133c) is less sensitive and detects 500–1000 genome equivalents.¹⁰ Assays targeting single copy genes or amplifying longer fragments are less sensitive than those targeting repetitive sequences or shorter fragments particularly in paucibacillary specimens and samples containing degraded DNA.¹⁶ IS6110 is at least 50 fold more sensitive than 'short fragment' *devR* (Rv3133c) assay using purified DNA.¹¹ However, *devR* (Rv3133c) is unlikely to be missing from MTB genome.

The USP methodology followed was found to be efficient for processing all samples and was compatible with smear examination, culture and genotypic assays, as proven by similar studies.⁸ In this study, the sensitivity of phenotypic methods (ZN, LJ and BACTEC 460) was 89.29%, whereas that of IS6110 was 94.64% and 'short fragment' *devR* (Rv3133c) was 80.36%. While genotypic assays were more sensitive compared to phenotypic methods; amongst genotypic methods, IS6110 was found to be more sensitive than 'short fragment' *devR* (Rv3133c) as concluded by similar studies.⁷ Samples negative by phenotypic methods and positive by genotypic methods reflects increased sensitivity of genotypic methods though false positivity can be attributed to uneven distribution in aliquots apportioned for testing, suboptimal copy number and possible cross contamination.

Conclusion

Sample processing by USP methodology is efficient for both phenotypic and genotypic testing. Along with phenotypic methods like ZN staining, simultaneous BACTEC 460 culture and genotypic methods targeting IS6110 and *devR* (Rv3133c) are likely to increase the yield of positive samples. The increased sensitivity of IS6110 may be attributed to presence of multi copy fragments in MTB whereas positivity by 'short fragment' *devR* (Rv3133c) in IS6110 negative samples may be due to conserved pattern of 'short fragment' *devR* (Rv3133c). The combined results of IS6110 and 'short fragment' *devR* (Rv3133c) may result in higher sensitivity in detecting MTB positive cases than using them alone.

Intellectual contributions of authors

Study concept: Brig A.K. Sahni, Wg Cdr S.P. Singh, Avinash Kumar

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Conflicts of interest

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